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(71)(72) Applicants and Inventors: GAROFF, Henrik [FI/SE]; Kroppåsbacken 4, S-126 58 Huddinge (SE). LI, Kejun [CN/SE]; Terapivägen 8B 2tr, S-141 55 Huddinge (SE).

(74) Agents: ONN, Thorsten et al.; AB Stockholms Patentbyrå, Zacco & Bruhn (publ), P.O. Box 23101, S-104 55 Stockholm (SE).

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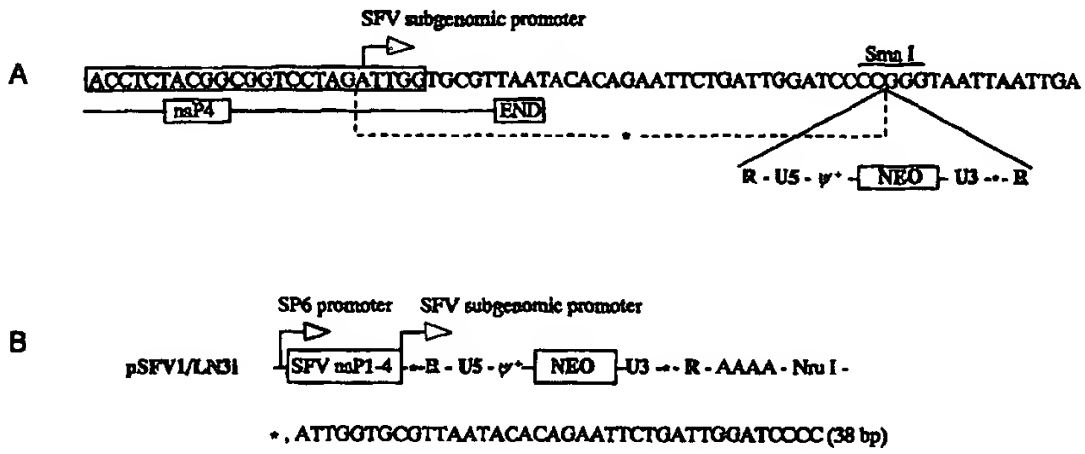

(57) Abstract

The present invention relates to RNA vectors comprising alphavirus RNA having inserted therein a recombinant retrovirus genome containing a foreign RNA sequence encoding a biologically active substance. The vectors of the present invention provide for the high level expression of the recombinant retrovirus genome directly in the cell cytoplasm of eukaryotic cells. When coexpressed with structural proteins of the retrovirus, the recombinant genome can efficiently be packaged into infectious recombinant retrovirus, also called retrovirus vectors, which can transduce the foreign RNA into recipient cells. Most importantly this cytoplasmic expression system facilitates the efficient production of vectors containing foreign RNA comprising genes in combination with introns or other control elements of gene expression. Such vectors have been impossible or very difficult to produce by the conventional nuclear expression systems because of RNA splicing.

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ALPHAVIRUS-RETROVIRUS VECTORS

Technical field

The present invention relates to the production of infectious recombinant retrovirus that can be used for the establishment of a stable expression of a gene in eukaryotic cells, for instance for the purpose of human gene therapy.

Background of the invention

In the classical type of human gene therapy it is desired to obtain a stable expression of a gene in somatic cells of the human body. In experiments used so far this has mostly been obtained by infecting human cells with retrovirus vectors. A retrovirus is an enveloped RNA virus which carry a reverse transcriptase, which converts its genome into dsDNA and further an intergrase, which catalyses the insertion of the genome into the DNA of host chromosomes (Luciw and Leung 1992). The integrated viral genome, or provirus, is copied by transcription into RNA molecules that are transported into the cytoplasm and then encapsidated into virus particles. When used as vectors, the proviral DNA is engineered and transfected into packaging cells (Miller and Rosman 1989; Hodgson 1996). The engeneered provirus is called the retrovirus DNA vector. It represents a recombinant retrovirus genome. In this DNA most or all of the gene regions encoding the retrovirus structural proteins and enzymes have been replaced with a foreign gene. The packaging cells represent a stably transformed cell line that produces the retrovirus structural proteins (e.g. the capsid protein gag and the membrane protein env) and enzymes (Miller 1990). When the retrovirus DNA vector is tranduced into the packaging cells, e.g. by transfection, it will be transcribed by the nuclear transcription machinery into RNA. This RNA is equivalent to the viral RNA. It is called the recombinant retroviral RNA or retrovirus RNA vector. The retroviral RNA is transported from the nucleus of the cell to the cytoplasm. In here it will be packaged into a recombinant retrovirus particle , that is a retrovirus vector, through the recognition of its encapsidation signal (a certain RNA sequence) by the virus structural proteins. Like wild-type retrovirus, the retroviral vectors are able to infect target cells and facilitate recombinant genome integration into the chromosomes. However, opposite to the wild-type retrovirus, the recombinant genome cannot express viral structural proteins for particle production. It can only express the foreign gene. In this way the recombinant retrovirus can be used as a vector for the expression of a foreign gene for instance in human cells. Today most retrovirus vectors are based on the Moloney Mouse Leukemia Virus (MLV) but vectors based on the Human Immunodeficiency Virus (HIV)-1 has also been developed (Miller and Rosman 1989; Naldini, Blömer et al. 1996; Zufferey, Nagy et al. 1997).

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While the principle of using retrovirus based vectors for human gene therapy has been proven experimentally, its practical use is still combined with many problems. These are among others related to (1) the low production level of vectors in present production systems, (2) instability of vector preparation, (3) sensitivity of vector preparation to complement attack, (4) the lack of cell targeting specifity of the particles, (5) the difficulty in infecting nondividing cells, (6) insufficient expression level of foreign gene in transduced cells, (7) lack of tissue and cell differentiation specific gene expression and (8) short duration of the expression of the foreign gene.

The latter three problems, which relate to the mode of gene expression, are mainly a consequence of the fact that the retrovirus vector system is in practice limited to transduce cDNA forms of processed mRNAs. These minigenes are usually not compatible with efficient, durable and controlable gene expression in cells. For this, other elements of natural genes like introns, enhancers and locus control elements are required. This has today been clearly demonstrated in a large number of studies (Grosveld, van Assendelft et al. 1987; Konieczny and Emerson 1987; Rossi and de Crombrugghe 1987; Bender, Miller et al. 1988; Brinster, Allen et al. 1988; Buchman and Berg 1988; Chang, Liu et al. 1992; Jonsson, Foresman et al. 1992). Such elements cannot however be incorperated into the retrovirus vector because they result in splicing of the recombinant retrovirus RNA when this is produced in the nucleus of the producer cell. For instance if an intron is introduced together with a foreing gene into a retrovirus vector it will efficiently be removed by splicing (Shimotohno and Temin 1982; Sorge and Hughes 1982). Attempts have been made to "hide" the splice signals by inserting foreign genes with introns in reverse orientation into the provirus but this has usually created new furtuitous splice signals in the reversed sequences (Leboulch, Huang et al. 1994; Jonsson, Habel et al. 1995). Similar problems have been encountered when including various other control elements for gene expression into the retrovirus vector (McIvor 1990).

The other problems of the contemporary retrovirus vector systems are related to the low synthesis rate of the viral structural proteins in producer cells, the features of the retrovirus assembly process and the functions of the retroviral structural proteins. Thus it should in principle be possible to obtain particles with increased stability and new and more purposeful functions by redesigning the viral structural proteins. For instance the cell-targeting function of the vector might be changed by engeneering of the env protein. To be successful, this requires however the construction and testing of many different vector variants and hence also fast and convenient systems to produce retrovirus vectors. The establishment of whole series of different packaging cell lines for such purposes would be extremely time consuming. Therefore transient production systems of recombinant retrovirus particles have recently been developed (Landau and Littman 1992; Soneoka, Cannon et al. 1995). In these systems the genes for the retrovirus structural proteins and the retrovirus recombinant genome are cotransfected into cells

and recombinant retrovirus particles are produced as a result of transient nuclear coexpression of the recombinant retrovirus RNA and the mRNAs for the viral structural proteins and enzymes. Using these systems only about three days are required to make a preparation of recombinant retrovirus vectors. However the yield of vectors obtained by these systems is usually very low, especially if a three component gene mixture (the env gene, the gag-pol gene and the recombinant retrovirus DNA) is used for transfection.

The present invention provides alphavirus-retrovirus RNA-vectors which drive efficient production of infectious recombinant retrovirus particles when introduced into cell cytoplasm of eukaryotic cells. A preparation with a high concentration of recombinant retrovirus vectors can be produced by only 10 hr incubation of producer cells. Furthermore genes with introns, and other control elements of gene expression, can be encapsidated into the recombinant particles. The vectors are based on the genomic RNA molecule of an alphavirus. These RNA molecules are of plus (+) polarity and translated into the viral polymerase proteins at the onset of alphavirus infection. The polymerase replicates the viral genome and also transcribes its 3' end into the viral subgenome that functions as mRNA for the alphavirus structural proteins. The alphavirus expression is very efficient and leads to massproduction of viral RNA and proteins. Because of these properties, the alphavirus has been developed into "self-replicating" RNA-vectors for expression of foreign genes in eukaryotic cells (Xiong, Levis et al. 1989; Liljeström and Garoff 1991). In these vectors the foreign gene is inserted into the subgenomic region of the alphavirus. When the recombinant RNA is transfected into cell cytoplasm, it will be replicated and transcribed into recombinant subgenomes which will be translated into the foreign gene product. As an alternative to transfection, the recombinant alphavirus genomes can also be packaged into alphavirus particles and transduced into cells by virus infection. The recombinant particles are produced by coexpressing the recombinant alphavirus genome together with a "helper" variant of the alphavirus genome. The latter contains the complete alphavirus subgenome and its promoter region as well as all of the RNA elements which are required for RNA replication. However, it lacks RNA elements required for packaging. The major advantages with the alphavirus expression system are high level expression, fast and convenient usage, and the possibility to use the alphavirus particles to infect a wide range of host cells.

Accordingly, it is an object of the present invention to provide alphavirus-retrovirus RNA molecules (also called alphavirus-retrovirus RNA-vectors) which can be transcribed into recombinant retrovirus genomes (with or without introns and other control elements of gene expression) that, in turn, can be packaged into infectious recombinant retrovirus particles also called retrovirus vectors.

It is another object of the present invention to provide recombinant alphavirus particles containing aforementioned alphavirus-retrovirus RNA molecules.

It is yet another object of the present invention to provide methods and compositions which permit the replication of the aforementioned recombinant alphavirus-retrovirus RNA in cells, its transcription into recombinant retrovirus genomes and the packaging of the latter genomes into infectious recombinant retrovirus particles.

We could foresee two major difficulties in our attempts to produce functional retrovirus vectors by using alphavirus-retrovirus RNA vectors. Firstly, in all retrovirus packaging systems described so far the retrovirus genes are produced in nucleus and not in cytoplasm as is the case when using alphavirus expression vectors. If a nuclear localization of the retrovirus genome is required for its efficient packaging, the alphavirus driven expression system will most likely be inappropriate. Secondly, it is not possible to produce an authentic retrovirus genome in the form of an alphavirus subgenome because the latter requires some alphavirus specific sequences at its 5' and 3' ends. These are a 5' end sequence, which constitutes both the 3' region of the alphavirus subgenome-promoter and the coding sequence of nonstructural protein 4, and a 3' end sequence that constitutes a viral RNA replication signal (Strauss and Strauss 1994). Thus, the addition of these sequences to 5' and 3' ends of the retrovirus genome is necessary for its expression by the alphavirus vector. It was not clear to us to what extent such sequence addition influences retrovirus genome packaging into particles, reverse transcription, polymerization into double-stranded DNA, chromosome integration and expression.

Summary of the invention

The present invention relates to vectors comprising alphavirus RNA having inserted therein a recombinant retrovirus genome. In one embodiment of the present invention, the alphavirus RNA comprises a Semliki Forest virus (SFV) RNA and the recombinant retrovirus genome comprises a recombinant genome.

In another embodiment of the present invention, alphavirus RNA with an inserted recombinant retrovirus genome, containing a foreign gene with or without an intron (or some other control element for gene expression), is provided, which permit replication and packaging of said RNA into alphavirus particles in the presence of replication competent helper RNA, which encodes the structural proteins of the alphavirus.

In a yet another embodiment of the present invention, alphavirus RNA with an inserted recombinant retrovirus genome containing a foreign gene with or without an intron (or some other control element for gene expression), is provided, which permit replication of the said

retrovirus genome and its packaging into recombinant retrovirus particles in the presence of replication competent helper RNAs, which encode the retrovirus structural proteins.

In a further embodiment of the present invention, genetically altered alphaviruses and/or cells comprising alphavirus RNA having inserted therein a recombinant retrovirus genome, containing a foreign gene with or without an intron (or some other control element for gene expression), is provided.

Brief description of the drawings

Fig.1 A depicts the DNA sequence near the SFV subgenomic promoter. The MLV recombinant genome is inserted into the *Sma* I site of pSFV1-Nru I vector.

Fig.1 B depicts the pSFV1/LN3i construct. Only the SFV recombinant region of the construct is shown. This region extends from the SP6 promoter (open arrow) to the *Nru* I site. The construct contains, in 5' to 3' direction, (i) the 5' replication signals of SFV RNA, (ii) genes encoding the SFV replication complex (nonstructural proteins, nsp, 1-4), (iii) the internal subgenomic promoter of SFV (solid arrow), (iv) the recombinant MLV genome, including the 5' R-U5, the encapsidation signal (ψ^+), the neo^R gene and the 3' U3-R sequences as represented in the MLV vector pLN (Miller and Rosman 1989) and 38 SFV-specific bases (denoted with *) both before the 5' R region and between the 3' U3 and R region, (v) the 3' replication signals of the SFV RNA and (vi) the polyA tract of the SFV genome. Note that coding regions indicated are not to scale.

Fig.2 depicts the construction of plasmid pSFV1/LN3i. Relevant restriction endonuclease sites and engineering steps are indicated.

Fig.3 depicts RNA analysis of transfected cells. BHK-21 cells were transfected with SFV1/LN3i RNA (Lane 1), SFV1/gag-pol RNA (Lane 2), SFV1/Pr80env RNA (Lane 3), or all three RNAs (Lane 4). Transfected cells were labeled with [^{14}C]uridine for 6 hr in the presence of actinomycin D. Cellular RNAs were isolated and separated on 0.7% agarose gels containing formaldehyde. Radiolabeled bands were visualized by autoradiography. The positions of the replicated genomic and the transcribed subgenomic RNAs are indicated.

Fig.4 depicts the cell-associated and extracellular protein analysis from BHK-21 cells cotransfected with SFV1/LN3i RNA, SFV1/gag-pol RNA, SFV1/AMenv RNA. lys: cell lysate, ip: cell lysate immunoprecipitation, med: medium, M: marker. The envelope protein products and gag protein products are indicated.

Fig.5 depicts that the gag precursor production is more efficient in cells transfected with SFV-C/gag-pol RNA than in cells transfected with SFV1/gag-pol RNA. The MLV specific Pr65gag, p30 and pp12 proteins are indicated.

Fig.6 depicts the construction of pSFV1/LN-U3insert. In pSFV1/LN-U3insert, the recombinant MLV genome (U3-R-U5-ψ^+-neo^R-U3-R) from pLN (Miller and Rosman 1989) was inserted between the *BamH* I and *Sma* I sites of plasmid pSFV1-Nru I plasmid (Fig. 6A and B). Note that the 35-base SFV sequence (denoted with *) which is flanking the recombinant MLV genome on its 5' side and which contains part of the SFV subgenomic promoter is also inserted into 3' U3 just after the sequence specificing for DNA integration (Fig. 6 B and C).

Fig.7 depicts the construction of the plasmid pSFV1-I-CAT. (A) is a schematic representation of the structure of the pCAT3-promoter vector. The engineering strategy of pSFV1-I-CAT is shown in (B). (C) is a schematic representation of the recombined SFV region of SFV1-I-CAT. The CAT gene with the intron was isolated from the pCAT3-promoter vector (Promega) (Fig. 7A) and inserted as a Bgl II-Bam HI fragment into an pSFV1/LN3i. To facilitate this, a unique Bam H1 site was created into the latter plasmid at a position after the neo^R gene region. The intermediate was denoted pSFV1/LN3i (BNNP). This required first the removal of two existing Bam H1 sites followed by the insertion of a new site. Fig. 7B shows schematically the engineering strategy and Fig. 3C the functional gene regions of the recombined SFV part in pSFV1-I-CAT. The intronless pSFV1-CAT was derived from pSFV1-I-CAT by excising the intron containing DNA fragment with Hind III.

Fig.8 depicts CAT activity in NIH 3T3 cells infected with recombinant retrovirus particles containing the Cat gene with (SFV1-I-CAT) or without intron (SFV1-CAT). NIH 3T3 cells were plated into 60 mm dishes at 5 x 10^5 cells/dish 24 hr before infection. The cells were infected with 1 x 10^5 recombinant retrovirus particles. CAT activity was tested using CAT Enzyme Assay System With Reporter Lysis Buffer (Promega) 52 hr after infection. In brief, the cells extracts were incubated in a reaction mix containing [^{14}C]labeled chloramphenicol and n-Butyryl Coenzyme A at 37°C for 16 hr. CAT transfers the n-butyryl moiety of the cofactor to chloramphenicol. The reaction products were extracted with 300µl of xylene. The n-butyryl chloramphenicol partitions mainly into the xylene phase, while unmodified chloramphenicol remains prodominantly in the aqueous phase. The xylene phase was mixed with 3ml scintillant and counted in the liquid scintillation counter. The cpm measured in each sample represents the butyrylated chloramphenicol.

Detailed description of the invention

The RNA vectors of the present invention provide a means for replicating and expressing recombinant retrovirus genomes independent of the host nucleus in the cytoplasm of several different types of eukaryotic cells. The expressed recombinant retrovirus genomes can be packaged into infectious recombinant retrovirus particles, also referred to as "retrovirus vectors", if coexpressed with retrovirus structural proteins. The recombinant retrovirus genome refers to a "retrovirus RNA genome" that contains all those RNA elements that are required in *cis* for retrovirus genome encapsidation into retrovirus particles, reverse transcription, dsDNA synthesis, retrovirus DNA integration into host chromosomes and retrovirus DNA transcription in cell nucleus. Further in the recombinant retrovirus genome, the region encoding the retrovirus structural proteins has been exchanged with a heterologous sequence encoding a foreign protein. The foreign gene can also be linked to an intron or some other control element of gene expression. Because of the cytoplasmic mode of RNA replication in this expression system the latter sequences will not be subjected to nuclear RNA processing events like splicing. The recombinant retrovirus particle refers to a particle in which a recombinant retrovirus genome (with or without an intron or some other control elements of gene expression) has been packaged into a retrovirus-like particle. This recombinant particle can mediate the transduction of the recombinant retrovirus genome into a new cell via the process of retrovirus infection.

The methods and compositions are briefly described below. For construction of the RNA vector we used the pSFV1-Nru1 plasmid into which we inserted the recombinant MLV genome, R-U5-ψ^+-neo^R-U3-R, from plasmid pLN (Miller and Rosman 1989) (Fig. 1). The pSFV1-NruI plasmid corresponds to the earlier described pSFV1 plasmid (Liljeström and Garoff 1991), but it contains a 527 base pair deletion between the *Stu* I and *Hind* III sites of pSFV1 and furthermore the *Spe* I site of pSFV1 has been changed into *Nru* I site. The insertion of the recombinant retrovirus genome into pSFV1-Nru I was made so that the recombinant retrovirus genome followed in 3' direction the promoter region for the SFV subgenome (Fig. 1A). In this way the recombinant retrovirus genome is expressed instead of the viral subgenomic RNA. However, as the promoter region for the SFV subgenome overlaps with the extreme 5' region of the subgenomic transcript itself, the 5' end of the recombinant retrovirus gene cannot be joined directly at the transcription start site, but somewhat further down-stream (Strauss and Strauss 1994). We made the 5'-gene fusion at a point 38 bases down-stream from the transcription start site at a *Sma* I site in the plasmid pSFV1-Nru I plasmid. Thus, the transcribed recombinant retrovirus genome will contain 38-base SFV specific residues at its 5' end (see Fig.1A). This created another problem. According to the model for retroviral DNA synthesis by reverse transcriptase, a strong-stop DNA is synthesized near the 5' end of the retrovirus RNA

genome (Ramsey and Panganiban 1993). This strong-stop DNA then jumps to the 3' end, and the exposed R sequence hybridizes with the complementary R sequence at the 3' end of the retrovirus RNA genome for synthesizing the minus-strand DNA. The 38 SFV specific bases inserted in front of the 5' end R region would interrupt the minus-strand synthesis. To facilitate the conversion of the RNA into double-stranded DNA, we also inserted the same 38-base long SFV sequence between the 3' U3 and R regions (Fig. 1B). In all of these manipulations we used standard methods in molecular biology that can be used by anybody skilled in the art.

We realized that the 38-base SFV sequence in pSFV1/LN3i insert will also be present in the 5' end of the integrated recombinant retrovirus genome as well as in the 5' of the transcript that is made from the integrated genome (Ramsey and Panganiban 1993). This is expected to have a negative influence on the expression efficiency of the recombinant gene. To avoid this problem, we have constructed the pSFV1/LN-U3insert. In this construct, the recombinant retrovirus genome (U3-R-U5-ψ^+-neo^R-U3-R) from pLN was inserted between the *BamH* I and *Sma* I sites of pSFV1-Nru I plasmid (see Fig. 6A and B). A 35-base SFV specific sequence which contains part of the SFV subgenome promoter region is located between the SFV transcription initation site and the start of the recombinant retrovirus genome. The same sequence was also inserted into the 3' U3 region of the recombinant retrovirus genome at a position just downstream of the region specifying for retrovirus DNA integration (Fig. 6B and C). In this case the double-stranded DNA synthesis process of the recombinant retrovirus will result in a DNA molecule that can be integrated into chromosomes so that no SFV specific sequences will be present in its 5' end and also no SFV sequences will be present in the 5' end of transcribed RNA.

Plasmid pSFV1/LN3i was used for transcription of corresponding RNA vector *in vitro*. This was done as described using SP6 polymerase (Liljeström and Garoff 1991). The RNA was transfected into BHK-21 cells and its replication in cell cytoplasm was followed by labeling with ^{14}C-uridine for 6 hr. Samples containing cytoplasmic RNA were analysed on a agarose gel. Fig.3, lane 1, shows that both full-sized and subgenomic RNA have been produced. This indicates that the SFV1/LN3i RNA vector can be used for the production of recombinant retrovirus genome molecules in cell cytoplasm. In this method we used RNA transfection for introducing the vector RNA into cell cytoplasm. An alternative method is to package this RNA into SFV particles using cotransfection with SFV-helper1 RNA (Liljeström and Garoff 1991) and then to infect the BHK-21 cells with the recombinant SFV particles.

The important question was whether the recombinant retrovirus genomes that were produced in the cytoplasm of the cells were actually competent for packaging into recombinant retrovirus particles. This was tested for the SFV1/LN3i RNA by cotransfecting this RNA and two other

SFV-RNA vectors that were expressing the retrovirus structural proteins and enzymes. The latter RNAs were transcribed form the plasmids pSFV-C/gag-pol (or pSFV1/gag-pol) and pSFV1/AMenv (or pSFV1/Pr80env). The pSFV1/gag-pol plasmid contains the coding-region of the gag-pol of MLV (Suomalainen and Garoff 1994). This is also present in pSFV-C/gag-pol. The latter plasmid contains in addition the SFV capsid coding-region in front of gag-pol. In the transcribed C-gag-pol RNA, the C-region specifies increased translation efficiency as compared to gag-pol mRNA (Sjöberg, Suomalainen et al. 1994). The pSFV1/Pr80env contains the coding region of the homologous (ecotropic) MLV env precursor protein, and the SFV1/AMenv contains the coding region of the heterologous amphotropic env precursor protein. The latter envelope protein has the capacity to target the recombinant retrovirus particle to a broad range of animal host cells including human cells, whereas the ecotropic env only recognizes mouse cells. The protein synthesis in cells cotransfected with SFV1/LN3i, SFV-C/gag-pol and SFV1/AMenv RNAs was followed by metabolic labelling with [^{35}S]methionine. The results of a pulse-labelling experiment is shown in Fig.4. This shows that all retrovirus structural proteins have been expressed in the cells. The formation of virus particles was followed by analysis of the media from the cotransfected cells. Particles with correct protein compositions were found (Fig.4). The infectivity of the particles was studied by using the media containing the particles to infect NIH 3T3 cells and then selecting for Neo^R transformants with G418. The results (Table 1, p.15) showed that infectious particles were formed by our production procedure. This is a significant finding since it indicates that (1) a recombinant retrovirus genome that has been produced in cell cytoplasm, and not in the nucleus as during wild-type retrovirus infection, can be packaged into infectious retrovirus particles; and (2) insertion of SFV-derived RNA sequences in the subgenomic recombinant retrovirus RNA molecule is compatible with efficient recombinant retrovirus RNA packaging into recombinant retrovirus particles, reverse transcription, dsDNA synthesis, integration of retrovirus DNA into host chromosomes and expression of the integrated gene. Most importantly the time course of recombinant particle production (Table 1) shows that, when using RNA combinations including SFV-C/gag-pol RNA, not more than 10 h inbucation is required for the generation of a vector preparation with more than 10^6 particles/ml of culture medium. This is true for recombinant retrovirus particles with both ecotropic and amphoptropic env proteins. The analyses of the media from the three subsequent 5 h incubations showed that 2-4 x 10^6 particles were released during each of the incubation intervals. These concentrations of recombinant retrovirus particles are very high and corresponds to the highest ones reported for recombinant retrovirus vectors that have been produced by other stable or transient producer cell systems (Miller and Rosman 1989; Landau and Littman 1992; Pear, Nolan et al. 1993; Finer, Dull et al. 1994; Soneoka, Cannon et al. 1995).

Similar studies were performed with RNA made from pSFV1/LN-U3insert. The titer of the corresponding recombinant retrovirus preparation was approximately the same as that one which was obtained with the RNA from pSFV1-LN3i.

To test whether a gene with an intron could be encapsidated into the recombinant retrovirus particles and further whether these could be used for gene transduction we inserted the chloramphenicol acetyltranferase (CAT) gene with an intron into SFV1/LN3i (Fig.7). The resulting plasmid was called pSFV1-I-CAT. As control we used a corresponding intron free construction (pSFV1-CAT). In order to produce retroviral particles that contain the CAT gene with or without the intron we first transcribed SFV1-I-CAT and SFV1-CAT RNA in vitro from the corresponding plasmids. Each RNA was then transfected into BHK-21 cells together with the SFV-C/gag-pol and the SFV1-env RNAs. The latter two RNAs specifed gag-pol and env precursor production. The cells were incubated for 10-15 h after transfection and the media was collected. The released recombinant retroviruses were then used to transduce CAT genes into NIH 3T3 cells. The CAT activity of cells was measured using a standard CAT assay after 52 hours (Fig.8). Very high CAT activity was found in the cells infected with vectors containing CAT gene with the intron whereas very low activity was found in the cells transfected with the intronless vector. Thus, this shows that the intron containing CAT gene was successfully transduced with the retrovirus vector into the recipient cells and that it resulted in efficient CAT expression.

When considering to use recombinant retrovirus vectors for gene therapy in humans it is important to assess the safety risks. The major risk with recombinant retrovirus vector preparations is contamination by replication competent retrovirus particles. A replication competent particle has aquired all retrovirus structural protein genes and hence it has the capacity to spread from cell to cell. Such particles can be generated in the producer cell through the process of RNA recombination. The possible generation of replication-competent particles in our production system was tested using a marker rescue assay (van Beusechem, Kukler et al. 1990). No replication-competent particles were found in a sample containing 2.6×10^6 infectious recombinant particles. We conclude that the alphavirus-retrovirus RNA vectors can be used for the expression of a recombinant retrovirus genome which can be packaged into infectious recombinant retrovirus vectors carrying either the amphotropic or ecotropic envelope proteins without detectable production of any replication competent particles.

Altogether we describe here a new cytoplasmic expression system for the production of retrovirus vectors. We show that this system facilitates the efficient packaging of intron containing genes into retrovirus vectors. We also show that such vectors, as expected, direct much more efficient gene expression than vectors carrying the corresponding gene without an intron. Although we have so far only demonstrated the suitability of this system for the

production of vectors that carry an intron associated CAT gene, there is every reason to believe that the system should be equally applicable to the production of vectors with other intron containing genes including such ones that are of therapeutic interest. For instance efficient and tissue specific expression of the β-globin gene has been obtained in cells transfected with a β-globin gene complex including an intron and certain locus control elements (Chang, Liu et al. 1992). With our sytem it should be possible to package this gene complex into retrovirus vectors at high titer and use them for the treatment of hemoglobin disorders like β-thalassemia. Similarly a factor IX gene-intron complex has been characterized that direct efficient factor IX expression (Kurachi, Hitomi et al. 1995). This should also be possible to package into retrovirus vectors using the system we have described in this disclosure. Such vectors could be useful for gene therapy of patients suffering from bleeding disorder hemophilia B (Christmas disease).

We show furthermore that our retrovirus vector production system is very fast and efficient: only 10 hr incubation of transfected cells is required to produce a preparation which contains a high concentration of vector particles (>10^6 particles/ml). The system allows for the convenient variation of the qualities of the packaging components and hence also the functions of the recombinant retrovirus particles. Therefore, this new retrovirus vector production system should meet the need for an efficient, fast and convenient production system of recombinant retrovirus particles. Its use should speed-up the engeneering of particles that are more suitable for specific gene therapy purposes.

In our present examples we have used SFV expression vectors for production of MLV vectors. Because of the great similarities among the various alphaviruses (Strauss and Strauss 1994) it is expected that any alphavirus expression vector (e.g. a Sindbis virus vector, (Xiong, Levis et al. 1989)) can be used for the production of a retrovirus vector. Similarly we have in our examples only shown how to produce MLV vectors using alphavirus vectors but it should be equally possible to use our system for the production of other retrovirus based vectors e.g. HIV-1 vectors (Naldini, Blömer et al. 1996; Zufferey, Nagy et al. 1997). Finally, it should be noted we have in our examples produced retroviral structural proteins and enzymes by SFV RNA vectors. While this is one major reason for obtaining high titered stocks of vectors it is evident that these packaging components can also be produced by other heterologous expression systems (both transient and stable ones).

Example 1

All restriction enzymes and DNA modifying enzymes were obtained from Promega (SDS, Falkenberg, Sweden), New England Biolabs (In Vitro AB, Stockholm, Sweden) and Stratagene (La Jolla, CA,USA) and used in accordance with manufacturers' instructions.

[^{35}S]Methionine was obtained from Amersham (Buckinghamshire, England). [^{14}C]Uridine was obtained from DuPont (Du Medical Scandinavia AB, Sollentuna, Sweden).
PCR primers were synthesized in Scandinavian Gene Synthesis AB (Köping, Sweden) and CyberGene AB (Huddinge, Sweden). Plasmid pSFV1/Pr80 env has been described in Suomalainen et al. (Suomalainen and Garoff 1994). Plasmid pLN has been described in Miller (Miller and Rosman 1989)

Example 2

This example demonstrates the construction of pSFV1/LN3i. The procedure is shown schematically in Fig.2. pSFV1/LN3i was made by inserting a recombinant MLV genome (R-U5-ψ^+-neo^R-U3-R) from pLN (Miller and Rosman 1989) into the *Sma* I site of pSFV1-Nru I plasmid vector (Fig.1 A). The recombinant retroviral genome in pSFV1/LN3i is flanked at the 5' end by 38 SFV-specific bases (part of which encodes the internal SFV promoter and the COOH-terminal region of the SFV nonstructural protein 4 (Liljeström and Garoff 1991; Strauss and Strauss 1994). In order to facilitate the conversion of the RNA into double-stranded DNA, we inserted the same 38-base long SFV sequence between the 3' U3 and R regions. This was done by fusion-PCR using Vent DNA polymerase (New England BioLabs). The following primers were used in the fusion PCR reaction:
primer A: 5' GCTCTAGAGAACCATCAGATG 3' (21 mer)
primerB: 5'GGGGATCCAATCAGAATTCTGTGTATTAACGCACCAAT
CCCGAGTGAGGGGTTGTGGGCT 3' (60 mer)
primer C: 5'ATTGGTGCGTTAATACACAGAATTCTGATTGGATCCCC
GCGCCAGTCCTCCGATTGACTG 3' (60 mer)
primer D: 5' CCCAAGCTTTGCAACTGCAAGAGGGTTTA 3' (29 mer)
The pLN/*EcoR* I fragments containing the 3'LTR were used as the template DNA. The reaction mixture was denatured at 94^{0}C for 45 s, annealed ar 50^{0}C for 45 s, and elongated at 72^{0}C for 1 min. After 25 cycles of amplification, the fusion PCR products were purified using Wizard PCR Preps DNA Purification System (Promega, SDS, Falkenberg, Sweden). The fusion PCR fragment was digested with *Hind* III and *Xba* I, and subcloned between *Hind* III and *Xba* I sites of pUC18 plasmid vector, making pUC18/insert plasmid. The fusion PCR fragment was verified by sequence analysis. The pUC18/insert plasmid was cut with *Hind* III, filled with DNA polymerase I large (Klenow) fragment and then cut with *Xba* I. A 262 bp *Hind* III (blunt) - *Xba* I fragment was isolated. The pLN plasmid was cut with *Asc* I, filled with DNA polymerase I large (Klenow) fragment and then cut with *Xba* I. The 2221 bp *Asc* I (blunt) - *Xba* I fragment was isolated. The pSFV1/LN3i was made by ligating the pLN/*Asc* I (blunt) - *Xba* I fragment and pUC18/insert *Hind* III (blunt) - *Xba* I fragment into *Sma* I cut of pSFV1-Nru I, as shown in Fig.2.

Example 3

This example demonstrates the constructions of pSFV1/gag-pol and SFV-C/gag-pol. Plasmid pSFV1/gag-pol contains the coding sequence of MLV retroviral structural precursor protein gag and the fusion protein gag-pol. The pol-part of the latter is the precursor for all viral enzymes. In plasmid pSFV-C/gag-pol, the translation enhancing RNA sequence of the SFV capsid gene was inserted in front of the gag-pol gene in pSFV1/gag-pol. pSFV1/gag-pol was made by inserting the MLV gag-pol cDNA from pNCA (Colicelli and Goff 1988) into the *Bam*HI site of pSFV1. The two *Spe* I sites in the gag-pol cDNA were removed by site-directed mutagenesis, using the oligonucleotide 5'GGGGGGTTGTTTGACGAGTGCCTCTACTGCATGGGGGG CCAGAATGACGAGTGGCTGTCCCATGGT 3' (Su and El-Gewely 1988). pSFV-C/gag-pol was made by ligating the *Not* I - *Bsm* I fragment (14410 bp) of pSFV-1/gag-pol and the *Not* I - *Bsm* I fragment (2723 bp) of pSFV-C/Pr65gag (Suomalainen and Garoff 1994).

Example 4

This example demonstrates the construction of pSFV1/AMenv. Plasmid pSFV1/AMenv contains the coding sequence of the murine amphotropic virus (4070A) envelope protein. The amphotropic envelope gene fragment from pPAM3 (Miller and Buttimore 1986) was first inserted into pUC18 by subcloning steps to make pUC18/AMenv. The plasmid pSFV1/AMenv was made by inserting the *Sma* I- *Hpa* I fragment (1976bp) from pUC18/AMenv into the *Sma* I site of pSFV1-Nru I.

Example 5

This example demonstrates the construction of pSFV1-Nru I. Plasmid pSFV1 [Liljeström, 1991 #15] was cleaved with *Stu* I and *Hind* III and the large fragment was filled with DNA polymerase I large (Klenow) fragment and ligated. The deleted plasmid molecule was cloned and used for *in vitro* mutagenesis. In this step, the *Spe* I recognization sequence (ACTAGT) was changed to that of *Nru* I (TCGCGA). This created the plasmid pSFV1-Nru I.

Example 6

This example demonstrates the replication of SFV1/LN3i and transcription of recombinant retrovirus RNA. Run-off transcripts were produced *in vitro* from *Nru* I-linearized pSFV1/LN3i using SP6 RNA polymerase (Liljeström and Garoff 1991). RNA (20µl) was transfected into 8 x 10^6 BHK-21 cells (American Type Culture Collection, Rockville, Maryland,USA) by electroporation. Electroporation was carried out at room temperature by two consecutive pulses at 0.85 kV and 25µF, using Bio-Rad Gene Pulser apparatus (Richmond, California, USA). Transfected BHK-21 cells were plated onto 33mm culture dishes and incubated for 2 hr at 37°C. Media were removed and replaced with 1 ml aliquots of medium containing 1µg/ml actinomycin D (Sigma-Aldrich Sweden, Stockholm, Sweden). After incubation for 2 hr at 37°C, media were replaced with 1 ml aliquots of medium containing 1µg/ml actinomycin D and

75 Kbq [^{14}C]uridine (2.1GBq/mmol, DuPont, Du Medical Scandinavia AB, Sollentuna, Sweden). After incubation for 6 hr at 37^{0}C, cellular RNA was isolated using TRIzol Reagent (GIBCO, Life Technologies AB, Täby, Sweden) as described by the manufacturer. RNA was dissolved in RNase-free H_2O and subjected to electrophoresis through 0.7% agarose gels containing formaldehyde (Sambrook, Fritsch et al. 1989). Gels were dried, and radiolabeled RNA was visualized by autoradiography. As shown in Fig.3, lane 1, high levels of both the replicated genomic SFV1/LN3i RNA and the transcribed subgenomic SFV1/LN3i RNA were transcribed in trasfected cells. When the BHK-21 cells were cotransfected with SFV1/LN3i RNA and other two RNAs, SFV1/gag-pol RNA and SFV1/Pr80env RNA which contain the coding region of retrovirus gag-pol and env respectively, all of the genomic and subgenomic RNAs were produced in the cotransfected cells (Fig.3, lane 4). Lanes 2 and 3 show RNA production in cells transfected with SFV1/gag-pol RNA and SFV1/Pr80env RNA, respectively.

Example 7

This example demonstrates viral protein synthesis in cells cotransfected with SFV1/LN3i RNA, SFV1/gag-pol RNA and SFV1/AMenv RNA by electroporation. Transfected cells were added to 9 ml complete BHK-21 medium, plated onto three 33-mm culture dishes and incubated at 37^{0}C. At 8 hr post-electroporation, transfected cells were washed twice with phosphate-buffered saline (PBS) and starved by incubation at 37^{0}C for 30 min in 2 ml methionine-free minimum essential medium (MEM, GIBCO, Life Technologies AB, Täby, Sweden) supplemented with 20mM Hepes. Media were then replaced with 0.5 ml methionine-free MEM containing 100µCi of [^{35}S]methionine per ml. After a 30 min pulse, cells were washed twice with MEM containing 20mM Hepes and 150µg/ml of unlabeled methionine (chase medium). Incubation was then continued in chase medium for 3 hours. The culture media were collected, cell monolayers were washed once with PBS and then solubilized in 0.3 ml of lysis buffer [1% sodium dodecyl sulphate, (SDS), 10mM iodoacetamide]. Media samples and cell lysates were clarified by centrifugation (Eppendorf centrifuge, 6000 rpm, 6 min). Cell lysates (0.3 ml) were diluted to 3 ml with NET buffer (150mM NaCl, 1mM EDTA, 50mM Tris-HCl pH 7.5, 0.1% NP-40, 0.25% gelatine, 0.02% sodium azide). To immunoprecipitate MLV-specific proteins, 5µl of polyclonal pig anti-MLV antiserum (HC 185, Quality Biotech, Camden, New Jersey, USA) and 40µl of protein A-Sepharose (Pharmacia, Uppsala, Sweden) slurry [1:1 (v/v) in 10mM Tris-HCl] were added to 1ml diluted cell lysate, and samples were incubated overnight at 4^{0}C. Immunoprecipitates were washed as described previously [Wahlberg, 1989 #23], and analyzed by SDS-PAGE (12%) under reducing conditions. Extracellular particles in media samples were pelleted through a 20% sucrose cushion (17,000 rpm, 2 hr, 10^{0}C, Beckman JA18.1 rotor). Pellets were analyzed by SDS-PAGE as described above. Gels were dried and exposed to Fuji film (Fuji Photo Film Co., LTD., Tokyo, Japan). The results are shown in Fig.4. All of the retrovirus proteins were synthesized in transfected cells and incoporated into virus-like particles. The gag precursor protein (Pr65) was observed in cell lysate and virus-like

particles. Most of the Pr65 was cleaved into the mature products, p30 and pp12. Two additional gag products, p15 (matrix protein) and p10 (nucleocapsid protein), are not visible because they lack methionines. The amphotropic envelope precursor protein (Pr85) in cell lysate was cleaved into surface proteins (gp70) and transmembrane proteins (p15E) by cellular protease. Only gp70 and p15E were incoporated into virus-like particles. The p15E was cleaved into p12E by viral protease.

Example 8

This example demonstrates that the expression of gag-pol products in the cells transfected with SFV-C/gag-pol RNA is much higher than in the cells transfected with SFV1/gag-pol RNA. BHK-21 cells were transfected with 20 µl of SFV-C/gag-pol RNA or 20 µl of SFV1/gag-pol RNA by electroporation. The transfected cells were pulsed for 30 min and chased for 15 min to 2 hr as decribed above. The cell-associated and extracellular MLV proteins were analyzed by SDS-PAGE(12%) under reducing condition. The results are shown in Fig.5. About 5-fold more gag-pol products were produced in the cells transfected with SFV-C/gag-pol RNA, as compared with that were produce in the cells transfected with SFV1/gag-pol RNA.

Example 9

This example demonstrates that infectious recombinant retrovirus particles is produced by cells cotransfected with SFV1/LN3i RNA, SFV1/gag-pol RNA (or SFV-C/gag-pol RNA), and SFV1/Pe80env RNA (or SFV1/AMenv RNA). The transfected BHK-21 cells were diluted into 9 ml complete BHK medium, and 6 ml of the cell suspension (containing 4 x 10^6 living cells) was plated onto a 60-mm culture dish (Nunclon, Roskilde, Denmark). The cells were incubated at 37°C, and the media were harvested at 5 hr interval from the same dish and replaced with 2 ml aliquots of fresh complete BHK-medium. The media were passed through a 0.45µm filter and stored at -130°C. *Neo*^{R}-transduction-competent retrovirus particles were titrated on NIH 3T3 cells. Therefore, NIH 3T3 cells were seeded at 5 x 10^5 cells per dish (60-mm) on day one. On day two, 1 ml aliquots of 10-fold serial dilutions of media samples were added to cell monolayers in the presence of 4µg/ml Polybrene (Sigma-Aldrich Sweden, Stockholm, Sweden). After incubation for 2 hr at 37°C, 1 ml aliquots of medium containing 4µg/ml Polybrene was added to each dish, and incubation was continued at 37°C. On day three, 24 hr after incubation, the cells were split 1:100 into selection medium containing 1mg/ml G418 (Geneticin, GIBCO, Life Technologies AB, Täby, Sweden). On day nine, the selection medium was replaced with fresh one. On day fifteen, G418-resistant colonies were stained with methylene blue (0.5% in 50% methanol) and counted. Virus titers are given as colony-forming units per ml (cfu/ml). They were calculated by multiplying the number of colonies with the dilution times and divided by 2 to account for cell doubling.

Table 1. Release of infectious recombinant retrovirus particles from transfected BHK-21 cells*

experiment	RNA †	$G418^R$ CFU‡/ml				
		0 - 5 hr	5 - 10 hr	10 - 15 hr	15 - 20 hr	20 -25 hr
1	SFV1/LN3i + SFV-C/gag-pol	—§	0	—	—	—
2	SFV1/LN3i + SFV1/Pr80env	—	0	—	—	—
3	SFV1/LN3i + SFV1/AMenv	—	0	—	—	—
4	SFV1/LN3i + SFV1/gag-pol + SFV1/Pr80env	3.7×10^4¶	8.0×10^5	1.1×10^6	8.5×10^5	6.5×10^5
5	SFV1/LN3i + SFV-C/gag-pol + SFV1/Pr80env	7.3×10^4	4.0×10^6	4.0×10^6	2.1×10^6	4.0×10^6
6	SFV1/LN3i + SFV-C/gag-pol + SFV1/AMenv	1.0×10	2.2×10^6	2.3×10^6	2.0×10^6	3.4×10^6

* In each experiment about 4×10^6transfected BHK-21 cells were plated into a 60mm culture dish and incubated at 37°C. The medium was collected and replaced at 5 hour intervals. Media samples were passed through 0.45µm filter and stored at -130 C before being used for titration.
† RNA used for transfection of BHK-21 cells
‡ CFU, colony forming units.
§ —, not analysed.
¶ 3T3 cells were incubated with diluted medium of transfected BHK-21 cells and then subjected to G418 selection. The numbers refer to resistant colonies formed after 12 days incubation.

The results are shown in Table 1. When SFV1/LN3i, SFV1/gag-pol and SFV/Pr80env RNAs were used to transfect the BKH-21 cell, 3.7×10^4 infectious particles were produced per ml during the first 5 hr incubation; this increased to 6.5×10^5-1.1×10^6 transduction competent particles per ml during the subsequent intervals. To increase the production of infectious particles, we used the pSFV-C/gag-pol construct which encodes the translation enhancing RNA sequence of the SFV capsid gene in front of the gag-pol gene. The expression of gag-pol products in cells transfected with SFV-C/gag-pol RNA is much higher than that of the corresponding products in SFV1/gag-pol RNA transfected cells. When the SFV-C/gag-pol RNA was used in a cotransfection/time course experiment, the production of infectious particles was considerably increased.The titer in most 5 hr-media samples was about 4×10^6 CFU/ml. To broaden the host range of target cells for the particles and to make the system suitable also for human cells, for instance in the context of gene therapy, we set up experiments for the production of MLV particles which were pseudotyped with the amphotropic envelope glycoprotein. Therefore, we cotransfected BHK-21 cells with RNAs transcribed from pSFV1/AMenv, pSFV1/LN3i and pSFV-C/gag-pol. The results in Table 1 show that a high titer stock was also obtained when the amphotropic env protein was used. Control experiments showed that no transduction competent particles were released into media of cells transfected with SFV1/LN3i and SFV-C/gag-pol, SFV1/LN3i and SFV1/Pr80env or SFV1/LN3i and SFV1/AMenv RNAs, respectively. These suggest that a retrovirus recombinant genome which has been produced in the cell cytoplasm using the SFV expression system, can be encapsidated by coexpressed packaging proteins into a high titer stock of transduction competent recombinant retrovirus particles.

Example 10

This example demonstrates that replication-competent particles were not detected. The possible presence of replication-competent particles in supernatant media was tested by a rescue assay. 3T3Zip*neo*SV(X)p cells, an NIH 3T3-derived cell line that harbours recombinant provirus consisting of the MLV LTRs, a packaging signal and the neo^R-gene were utilized in this assay: Transfection of these cells by the genes encoding the MLV gag-pol- and env-proteins results in the production of infectious particles containing the neo^R-recombinant genome. 3T3Zip*neo*SV(X)p cells were infected with the supernatant medium containing 2.6 x 10^6 infectious recombinant retrovirus particles in the presence of 4µg/ml Polybrene. The infected cells were passaged for 8 days. When the cells were about 50% confluent, the medium was replaced with fresh medium and the cells were incubated at 37°C. After a 24 hr incubation, the medium was collected, passed through 0.45µm filter and analyzed for the presence of neo^R-transduction-competent particles by titration on NIH 3T3 cells as described above. Media from uninfected 3T3Zip*neo*SV(X)p cells and cells infected with wild-type amphotropic retrovirus (4070A) were used as negative and positive controls, respectively. No colonies were obtained for media from 3T3Zip*neo*SV(X)p cells infected with either the recombinant particles produced by the SFV expression system, or the negative-control media. In contrast, about 4000 colonies were obtained using the positive-control media containing wild-type retrovirus.

Example 11

This example demonstrates the construction of pSFV1/LN-U3insert. pSFV1/LN-U3insert contains the recombinant retrovirus genome, U3-R-U5-ψ^+-neo^R-U3-R in the SFV subgenome region (Fig. 6). This was done as follows: (1) A 464 bp *Sfc* I - *Kpn* I fragment from the 3'LTR of pLN was cloned between *Bgl* II and *Kpn* I sites of pSP73, to make pSP73/U3. The *Sfc* I and *Bgl* II ends were filled with Klenow fragment. (2) A 2370 bp *Kpn* I - *Kpn* I fragment from pLN was cloned into the *Kpn* I site of pSP73/U3, to make pSP73/LN. (3) A gene segment corresponding to a 35 base fragment from 5 ' end of SFV subgenome was inserted into the 3'U3 region, just downstream to the site specifying for DNA integration, by fusion PCR (Horton, Hunt et al. 1989). Primers used for fusion PCR were upper 5' TGCTTGCCGAATATCATGGTG 3', lower primer 5' CCCAAGCTTTGCAACTGCAAGA GGGTTTA 3', and fusion primers 5' GATCCAATCAGAATTCTGTGTATTAACGCACCA ATGGTGGGGTCTTTCATTCCCC 3', 5' ATTGGTGCGTTAATACACAGAATTCTGATT GGATCTGTAGGTTTGGCAAGCTAGC 3'. The PCR reaction were carried out at 94°C for 45 s, 60°C for 45 s, and 78°C 2 min using the *Nco* I - *Nde* I fragments as the template DNA. After 25 cycles, the 862 bp fusion fragment s were purified using Wizard PCR Preps DNA Purification System (Promega, SDS, Falkenberg, Sweden). (4) The fusion PCR fragment was cut with *NgoM* I and *Hind* III and inserted between *NgoM* I and *Hind* III sites of pSP73/LN, to make pSP73/LN-U3insert. (5) pSP73/LN-U3insert was cut with *Hind* III, filling the end with Klenow fragment, and then cut with *Bgl* II. The 2973 bp *Bgl* II - *Hind* III (blunt) fragment was

isolated. The pSFV1/LN-U3insert was made by inserting the *Bgl* II - *Hind* III (blunt) fragment of pSP73/LN-U3insert between the *BamH* I and *Sma* I sites of pSFV1-Nru I.

Example 12

This example describes the construction of pSFV1/LN3i (BNNP). The plasmid was derived from pSFV1/LN3i by removing the two existing Bam HI sites and including a group of unique sites, also BamH I. The BamH I sites were removed by cutting pSFV1/LN3i with BamH I, filling with Klenow fragment, and religating. The resulting plasmid was called pSFV1/LN3i (-B). The group of new sites was inserted by fusion PCR. The sites included BamH I, Nde I, Nsi I and Pme I. Primers for fusion PCR were: 5' TGT CAA GAC CGA CCT GTC GC 3' (primer 1), 5' CCC AAG CTT TGC AAC TGC AAG AGG GTT TA 3' (primer 2), 5' GGA TCC ATA TGC ATG TTT AAA CGG ACT CTG GGG TTC GAT AAA 3' (primer 3) and GTT TAA ACA TGC ATA TGG ATC CCG CTC AGA AGA ACT CGT CAA 3' (primer 4). As template we used pSFV1/LN3i (-B). With the first two primers a 678 bp fragment containing the 3' end of the neo^R gene was synthesized. With primers 3 and 4 we synthesized a partial overlapping 641bp fragment containing the 3' LTR. The fusion PCR reaction resulted in a 1297 fusion fragment containing the unique sites. This was cut with BssH 2 and the 747 bp fragment isolated and inserted into BssH 2 cut pSFV1/LN3i (-B). The resulting plasmid was called pSFV1/LN3i(BNNP).

Example 13

Construction of pSFV1-I-CAT and pSFV1-CAT.

A CAT gene fragment plus an intron was isolated from pCAT3®-promoter vector (Promega, Catalog #E1861) by cleavages with Bgl II and Bam H1. The 1389 bp fragment was purified and inserted into pSFV1/LN3i(BNNP). This was done in a two fragment ligation with Bam H1 CAT and dephosphorylated pSFV1/LN3i (BNNP). The resulting plasmid was called pSFV1-I-CAT. The pSFV1-CAT was done similarly using the pCAT3®-promoter vector from which the intron had been removed. This was done by cleaving the latter plasmid with Hind III.

Example 14

Production of retrovirus vectors containing the CAT gene with or without the intron.

Recombinant retroviral particles containing the CAT gene with or without the intron was produced by cotransfection of SFV1-I-CAT RNA or SFV1-CAT RNA with both SFV-C/gag-pol RNA and SFV1-env RNA into BHK cells. After incubation for 10-15 h media were collected and used for titration of neo^R transduction competent particles. The titers were about $4x10^5$ particles/ml, for SFV1-I-CAT and $1x10^6$ particles/ml for SFV1-CAT.

Example 15

CAT expression efficiencies in cells transduced with recombinant retrovirus particles containing a CAT gene with and without an intron. About $1x10^6$ cells were infected with $1x10^5$ recombinant retrovirus particles. After 52 h lysates were prepared and CAT activity measured by using a standard assay (CAT Enzyme Assay System With Reporter Lysis Buffer, Promega). The results showed about 30 fold higher CAT activity in cells transduced with recombinant retrovirus particles containing CAT with an intron (Fig.8). Thus this example shows that an intron containing gene can be transduced into cells with our recombinant retrovirus particles and that this results in improved expression.

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Patent claims

1. An alphavirus-retrovirus RNA vector, which comprises replication competent, alphavirus genomic RNA containing in its subgenomic region recombinant retrovirus genomic RNA, which retrovirus genomic RNA comprises an exogenous RNA sequence encoding a biologically active substance, and from which vector, after introduction thereof in a host cell, said recombinant retrovirus genomic RNA with inserted exogenous RNA is transcribed to produce package competent recombinant genomic RNA which can be packaged into infectious recombinant retrovirus particles comprising said exogenous RNA.

2. An alphavirus-retrovirus vector of claim 1, wherein said exogenous RNA sequence contains an exogenous gene, suitably an exogenous gene comprising at least one of its introns and/or other control elements that are required for efficient expression of said gene, said control elements being endogenous and/or exogenous to said gene.

3. An alphavirus-retrovirus vector of claim 1 or 2 wherein the recombinant retrovirus genome has been inserted at a site down-stream of the alphavirus subgenome promoter and wherein the said recombinant retrovirus genome contains an insertion between the 3' U3 and R regions that corresponds in sequence to that part of the 5' alphavirus subgenome region that extends from the transcription start site of the alphavirus subgenome to the start of the recombinant retrovirus genome.

4. An alphavirus-retrovirus vector of claim 1 or 2 wherein the recombinant retrovirus genome contains an addition of a U3 sequence at its 5' end and an insertion corresponding to that part of the 5' alphavirus subgenome region that extends from the transcription start site of the alphavirus subgenome to the start of the recombinant retrovirus genome, in the 3' U3 region at a site just efter the DNA-integration specifying region.

5. An alphavirus-retrovirus vector of any of claim 1-4 wherein the alphavirus is SFV.

6. An alphavirus-RNA vector of claim 5 wherein the retrovirus is MLV.

7. An alphavirus-retrovirus vector of claim 1, wherein said alphavirus genomic RNA is derived from an alphavirus selected from the group consisting of Semliki Forest virus (SFV), Sindbis virus, Ross River virus and Venezuelan, Western and Eastern Equine Encephalitis viruses.

8. A DNA molecule which comprises DNA sequences complementary to an alphavirus-retrovirus RNA vector of any of claims 1-7, from which DNA molecule said alphavirus-retrovirus RNA vector can be transcribed.

9. A DNA molecule of claim 8 wherein the alphavirus sequences are represented by pSFV1-NruI and a recombinant MLV genome has been inserted into its polylinker region.

10. A recombinant alphavirus particle that contains an alphavirus-retrovirus RNA vector of any of claims 1-7.

11. A cell that contains an alphavirus-retrovirus RNA vector of claim 1.

12. A cell that contains a DNA molecule of claim 8.

13. A cell of claim 11 or 12, wherein said cell is a eukaryotic cell selected from the group consisting of avian; mammalian including human; amphibian; insect; and fish cells.

14. A method to produce infectious recombinant retrovirus particles, also called retrovirus vectors, that comprises the steps of: a) transfection of tissue culture cells with an alphavirus retrovirus RNA vector of any of claims 1-7 together with other alphavirus RNA vectors specifying retrovirus structural protein and enzyme production; b) incubation of cells; c) collection of media with released recombinant retrovirus particles.

15. A method to produce infectious recombinant retrovirus particles, also called retrovirus vectors, that comprises the steps of: a) transfection of tissue culture cells with an alphavirus retrovirus RNA vector of any of claims 1-7 together with other alphavirus RNA vectors specifying retrovirus structural protein and enzyme production, including the env precursor protein of the Amphotropic Murine Leukemia virus or another membrane protein that recognizes human cells; b) incubation of cells; c) collection of media with released recombinant retrovirus particles.

16. A method to produce infectious retrovirus particles, also called retrovirus vectors, that comprises the steps of a) infection of tissue culture cells with recombinant alphavirus particles containing an alphavirus-retrovirus RNA vector of any of claims 1-7 together with other recombinant alphavirus particles that contain alphavirus RNA vectors specifying the production of retrovirus structural protein and enzyme production; b) incubation of cells; c) collection of media with released recombinant retrovirus particles.

17. A method to use recombinant retrovirus particles, also called recombinant retrovirus vectors, for gene transduction into animal cells, including human ones, *in vitro* and *in vivo,* that comprises the steps of a) preparation of retrovirus vectors according to methods in claim 14 and 15; and b) using the recombinant retrovirus particles to infect cells.

18. A method to use recombinant retrovirus particles, also called recombinant retrovirus vectors for human gene therapy that comprises the steps of a) preparation of retrovirus vectors according to methods in claim 15; and b) using the recombinant retrovirus particles to infect cells.

FIG. 1

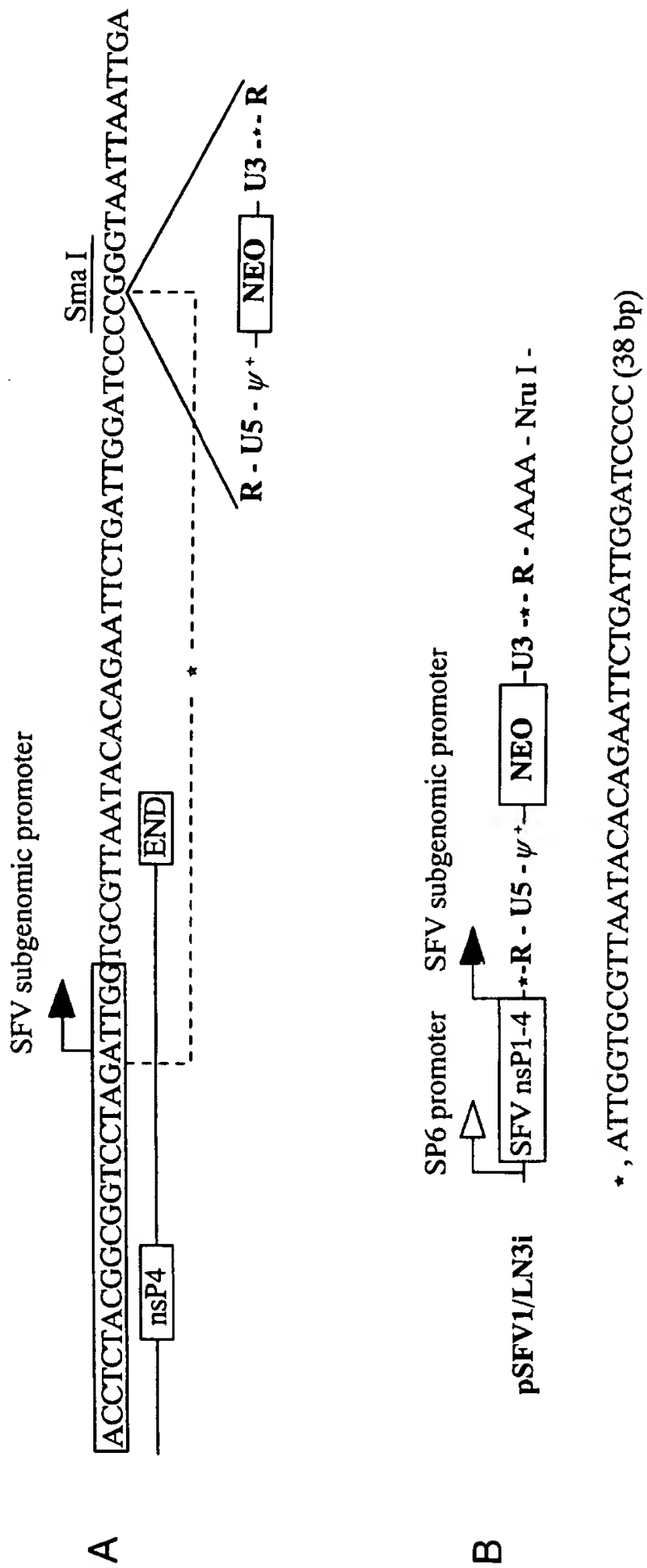
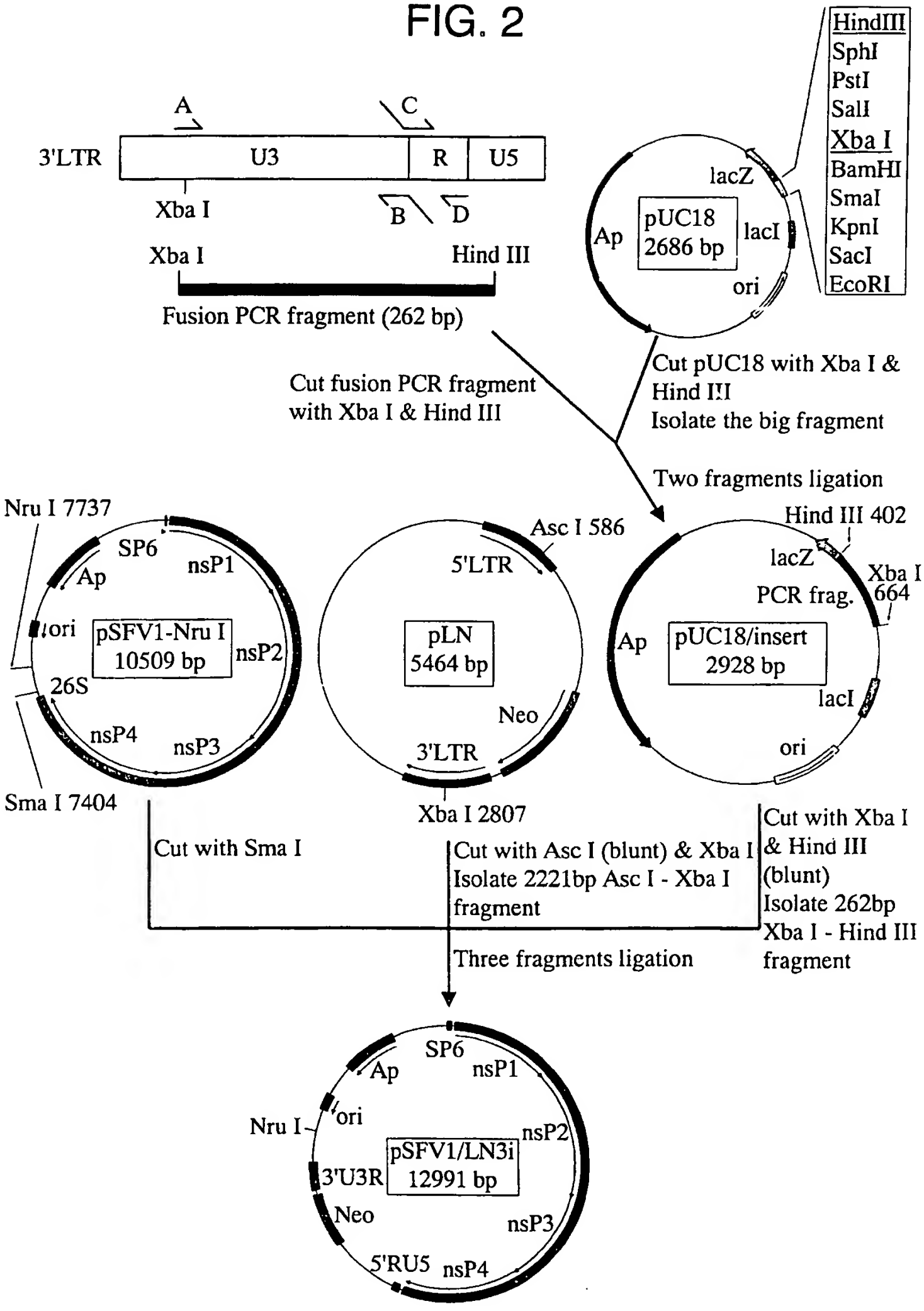

A
SFV subgenomic promoter
ACCTCTACGGCGGTCCTAGATTGGTGCGTTAATACACAG
nsP4
END
*
Sma I
CAGAATTCTGATTGGATCCCCGGGTAATTAATTGA
R - U5 - ψ+ NEO U3 - R
B
SP6 promoter
SFV subgenomic promoter
pSFV1/LN3i
SFV nsP1-4
*-R - U5 - ψ+ NEO U3 - R - AAAA - Nru I -
*, ATTGGTGCGTTAATACACAGAATTCTGATTGGATCCCC (38 bp)

FIG. 2


A
C
3'LTR
U3
R
U5
Xba I
B
D
Xba I
Hind III
Fusion PCR fragment (262 bp)
HindIII
SphI
PstI
SalI
Xba I
BamHI
SmaI
KpnI
SacI
EcoRI
lacZ
Ap
pUC18
2686 bp
lacI
ori
Cut fusion PCR fragment
with Xba I & Hind III
Cut pUC18 with Xba I &
Hind III
Isolate the big fragment
Two fragments ligation
Nru I 7737
SP6
nsP1
Ap
ori
pSFV1-Nru I
10509 bp
nsP2
26S
nsP4
nsP3
Sma I 7404
Asc I 586
5'LTR
pLN
5464 bp
Neo
3'LTR
Xba I 2807
Hind III 402
lacZ
Xba I
664
PCR frag.
Ap
pUC18/insert
2928 bp
lacI
ori
Cut with Sma I
Cut with Asc I (blunt) & Xba I
Isolate 2221bp Asc I - Xba I
fragment
Cut with Xba I
& Hind III
(blunt)
Isolate 262bp
Xba I - Hind III
fragment
Three fragments ligation
SP6
Ap
nsP1
Nru I
ori
nsP2
pSFV1/LN3i
12991 bp
3'U3R
Neo
nsP3
5'RU5
nsP4

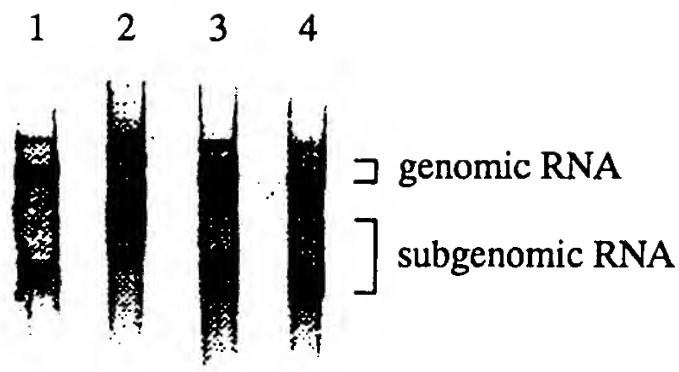


FIG. 3

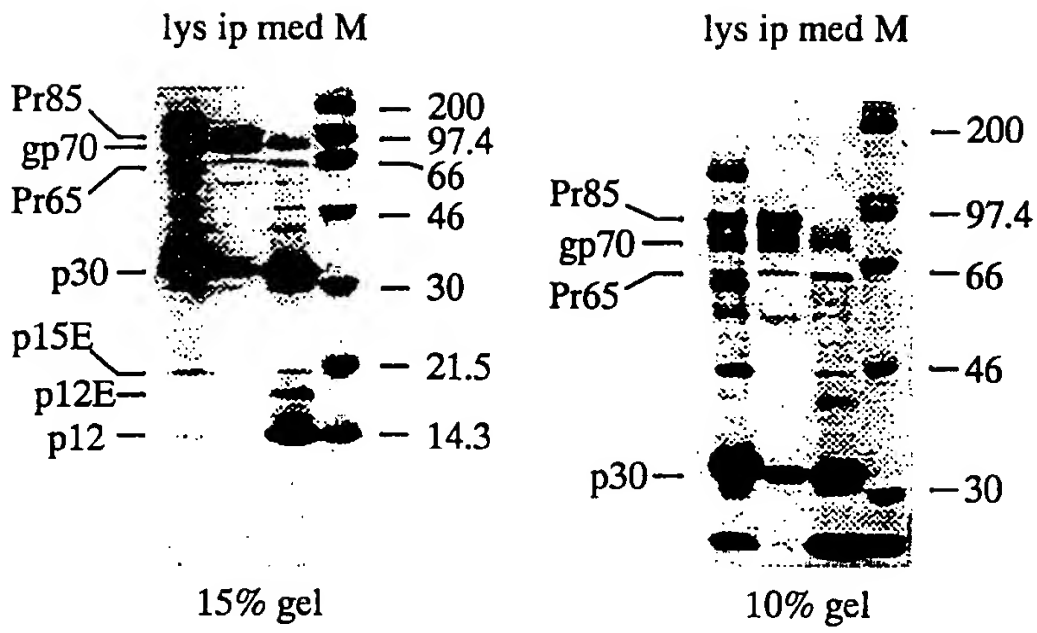

lys ip med M
Pr85
gp70
Pr65
p30
p15E
p12E
p12
200
97.4
66
46
30
21.5
14.3
15% gel
lys ip med M
Pr85
gp70
Pr65
p30
200
97.4
66
46
30
10% gel

FIG. 4

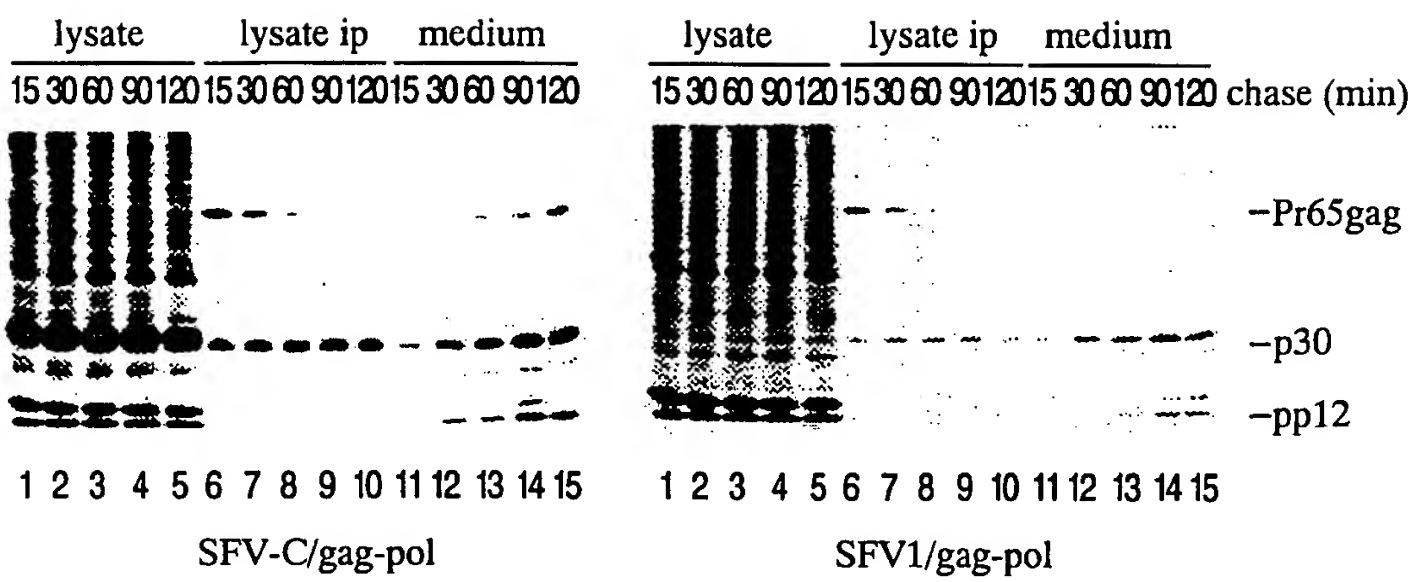
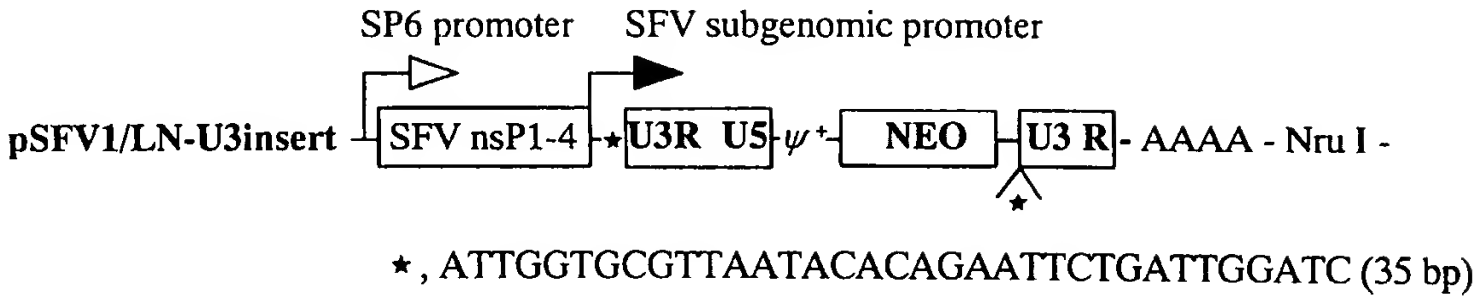

lysate
lysate ip
medium
15 30 60 90 120 15 30 60 90 120 15 30 60 90 120
lysate
lysate ip
medium
15 30 60 90 120 15 30 60 90 120 15 30 60 90 120 chase (min)
–Pr65gag
–p30
–pp12
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
SFV-C/gag-pol
SFV1/gag-pol

FIG. 5

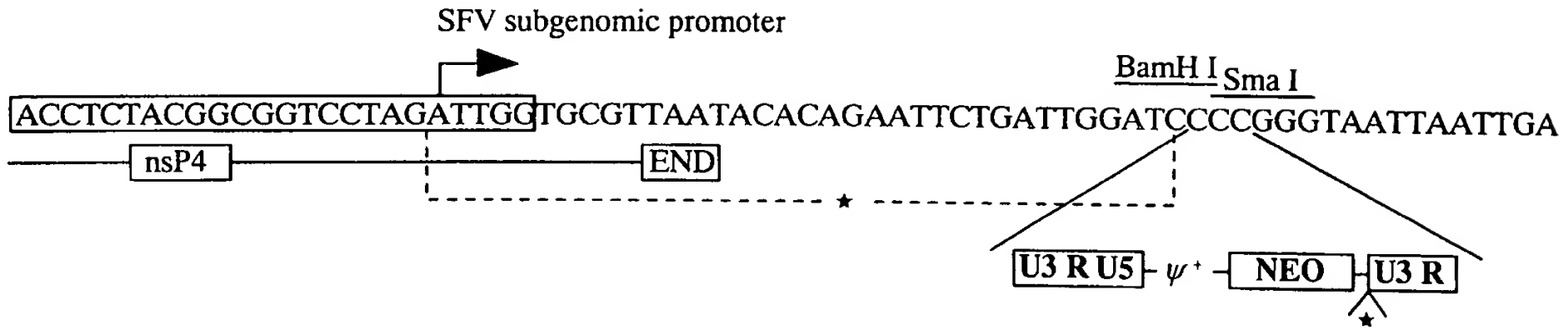
RECTIFIED SHEET (RULE 91)

FIG. 6

A



B



C

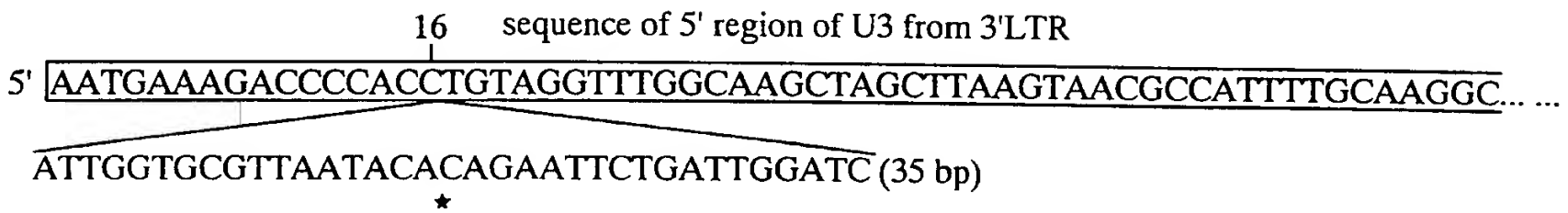
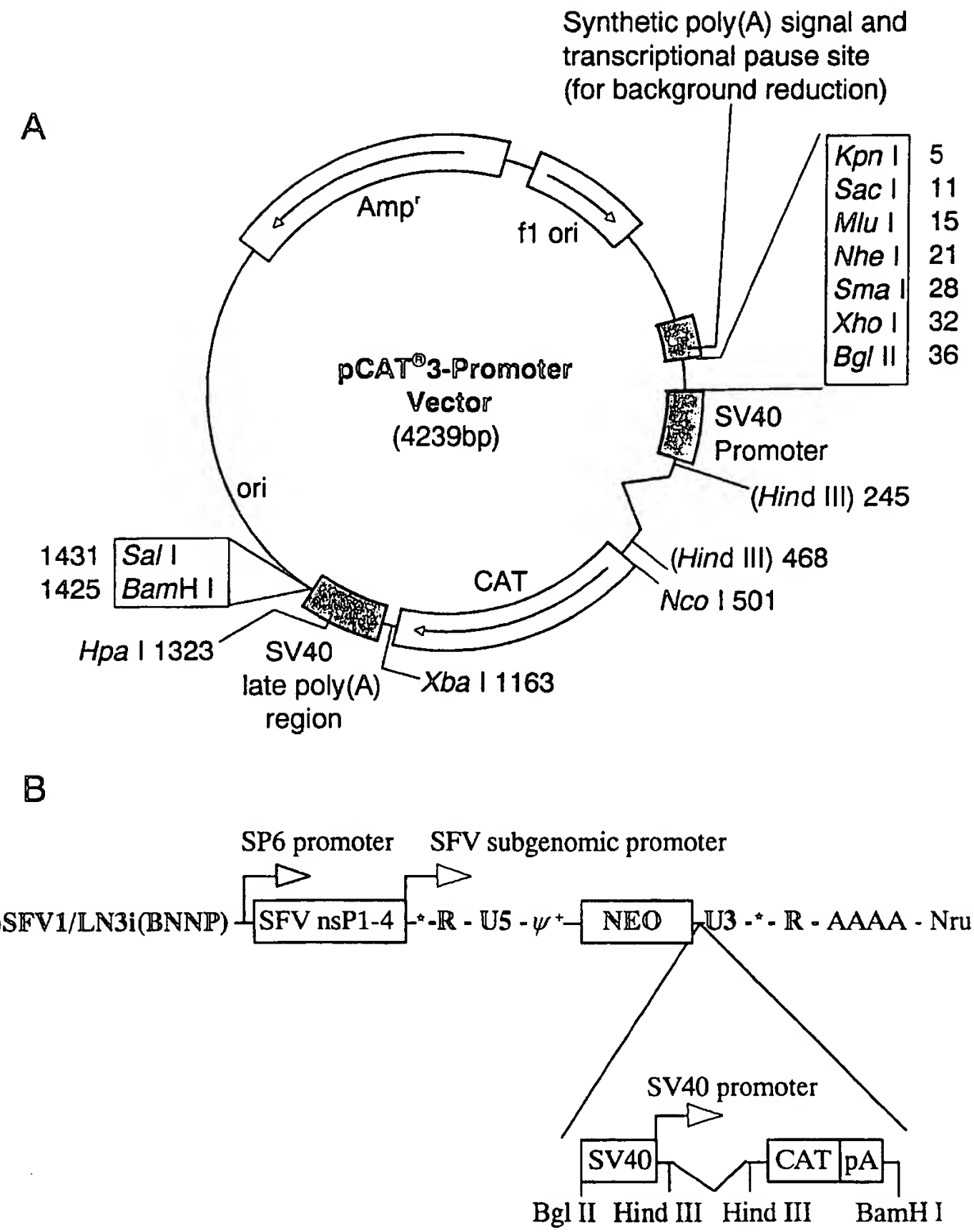


FIG. 7


A
Synthetic poly(A) signal and transcriptional pause site (for background reduction)
Kpn I 5
Sac I 11
Mlu I 15
Nhe I 21
Sma I 28
Xho I 32
Bgl II 36
Amp^r
f1 ori
pCAT®3-Promoter Vector (4239bp)
SV40 Promoter
(Hind III) 245
(Hind III) 468
Nco I 501
CAT
ori
1431 Sal I
1425 BamH I
Hpa I 1323
SV40 late poly(A) region
Xba I 1163
B
SP6 promoter
SFV subgenomic promoter
pSFV1/LN3i(BNNP)
SFV nsP1-4
-*-R - U5 -ψ+
NEO
U3 -*- R - AAAA - Nru I -
SV40 promoter
SV40
CAT
pA
Bgl II Hind III Hind III BamH I
C
SP6 promoter
SFV subgenomic promoter
SV40 promoter
SFV nsP1-4
-*-R - U5 -ψ+
NEO
SV40
CAT
pA
U3 -*- R - AAAA - Nru I -
Hind III Hind III

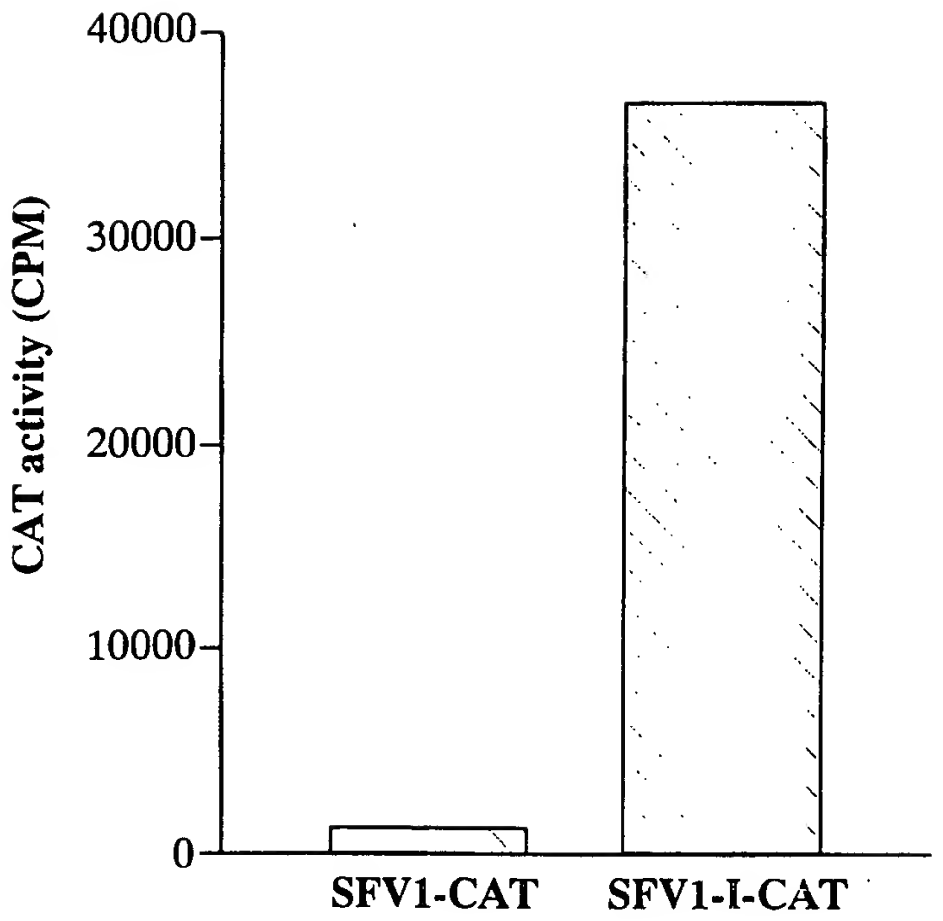

CAT activity (CPM)
40000
30000
20000
10000
0
SFV1-CAT
SFV1-I-CAT

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/01696

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/86, C12N 7/01, C12N 5/16, A61K 48/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, MEDLINE, BIOSIS, DBA, CA, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Proc.Natl.Acad.Sci., Volume 93, October 1996, Ke-Jun Li et al, "Prodcution of infectious recombinant Moloney murine leukemia virus particles in BHK cells using Semliki Forest virus-derived RNA expression vectors", page 11658 - page 11663	1-18
	--	
X	OECD Documents, Gene Delivery Systems, 1996, Peter Liljeström, "Alphavirus Vectors for Gene Delivery", page 109-118, see especially page 115, line 4-5	1-2,5-16
A		3-4,17-18
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 February 1998	16 -02- 1998
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

03/02/98

International application No.
PCT/SE 97/01696

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9210578 A	25/06/92	AU 656545 B	09/02/95
		AU 9078791 A	08/07/92
		CA 2098292 A	14/06/92
		EP 0561890 A	29/09/93
		FI 932678 D	00/00/00
		JP 6504198 T	19/05/94
		SE 9003978 D	00/00/00
WO 9617072 A2	06/06/96	AU 4594996 A	19/06/96
		EP 0797679 A	01/10/97

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/01696

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9210578 A (BIOPTION AB), 25 June 1992 (25.06.92), figure 8 ---	1-18
A	WO 9617072 A2 (CHIRON VIAGENE, INC.), 6 June 1996 (06.06.96) --- --------	1-18